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Generation of Specific Deoxynojirimycin-type Inhibitors of the Non-lysosomal Glucosylceramidase*

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ABSTRACT

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The existence of a non-lysosomal glucosylceramidase in human cells has been documented (van Weely, S., Brandsma, M., Strijland, A., Tager, J. M., and Aerts, J. M. F. G. (1993) *Biochim. Biophys. Acta* 1181, 55-62). Hypothetically, the activity of this enzyme, which is localized near the cell surface, may influence ceramide-mediated signaling processes. To obtain insight in the physiological importance of the non-lysosomal glucosylceramidase, the availability of specific inhibitors would be helpful. Here we report on the generation of hydrophobic deoxynojirimycin (DNM) derivatives that potently inhibit the enzyme. The inhibitors were designed on the basis of the known features of the non-lysosomal glucosylceramidase and consist of a DNM moiety, an *N*-alkyl spacer, and a large hydrophobic group that promotes insertion in membranes. In particular, *N*-(5-adamantane-1-yl-methoxy)pentyl)-DNM is a very powerful inhibitor of the non-lysosomal glucosylceramidase at nanomolar concentrations. At such concentrations, the lysosomal glucocerebrosidase and α-glucosidase, the glucosylceramide synthase, and the *N*-linked glycan-trimming α-glucosidases of the endoplasmic reticulum are not affected.

INTRODUCTION

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In recent years, the importance of ceramide as a second messenger has been recognized. It has become clear that the signal of some cytokines is mediated by changes in the intracellular concentration of this lipid (1, 2). For example, local changes in ceramide concentration in specific regions of the plasma membrane are crucial for the transduction of the signal exerted

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by tumor necrosis factor-α . Upon binding of the cytokine to its receptor, a sphingomyelinase catalyzes the conversion of sphingomyelin into phosphorylcholine and ceramide. The ceramide that is generated in this manner propagates the signal by activating specific protein kinases and phosphatases, resulting in the cellular response. This mechanism has been substantiated by the demonstration that the effects of tumor necrosis factor-α can be experimentally mimicked by the administration of a permeable ceramide with a truncated fatty acyl moiety or, alternatively, by the generation of ceramide at the cell surface by treatment of cells with a bacterial sphingomyelinase (see, for example, Ref. 2).

In the plasma membrane of cells, considerable amounts of ceramide are present as a building block in sphingomyelin and also in glycosphingolipids such as glucosylceramide. The latter lipids are not believed to play a role in ceramide-mediated signal transduction since their catabolism is thought to occur exclusively in lysosomes. The importance of intralysosomal glycosphingolipid catabolism is illustrated by the existence of inherited lysosomal storage disorders in which specific glycosphingolipids accumulate as the consequence of an inherited defect in some lysosomal glycosidases. One of the most common lipidoses is Gaucher's disease, a disorder caused by a deficiency in the lysosomal acid β-glucosidase, glucocerebrosidase[1] (EC 3.2.1.45), which hydrolyzes glucosylceramide into free glucose and ceramide (3). We discovered the existence of a non-lysosomal glucosylceramidase activity that is located near the cell surface (4). Besides its distinct subcellular localization, the non-lysosomal glucosylceramidase differs clearly in other aspects from the lysosomal glucocerebrosidase (4). In contrast to the latter enzyme, it is an integral membrane protein that is not deficient in Gaucher's disease patients. The two enzymes are also clearly distinct in their specificity toward artificial substrates, inhibitors, and activators (4). For example, the non-lysosomal glucosylceramidase is not able to hydrolyze artificial β-xylosidic substrates, contrary to glucocerebrosidase. Glucocerebrosidase is irreversibly inhibitable by conduritol B epoxide, in contrast to the glucosylceramidase, which is relatively insensitive to this compound. The lysosomal activator protein saposin C potently stimulates glucocerebrosidase in its enzyme activity, but is without effect on the non-lysosomal glucosylceramidase (4).

Earlier experiments with membrane suspensions have revealed that the ceramide that is formed by the non-lysosomal glucosylceramidase is efficiently converted into sphingomyelin, presumably by transfer of the phosphorylcholine moiety from phosphatidylcholine (4). The activity of the non-lysosomal glucosylceramidase might therefore result in (transient) changes in glucosylceramide, ceramide, phosphorylcholine, diacylglycerol, and sphingomyelin concentrations. Because of its localization close to the cell surface, a direct or indirect role for the non-lysosomal glucosylceramidase in the sphingolipid metabolism linked to ceramide-mediated signaling processes might be envisioned. To investigate this intriguing possibility, we have now developed novel specific inhibitors for the non-lysosomal glucosylceramidase. For this purpose, the available information on the membrane-bound feature of the enzyme and its relatively high affinity for inhibition by deoxynojirimycin-type compounds has been exploited. Here we report on the design, synthesis, and application of hydrophobic deoxynojirimycin analogues as specific inhibitors for the non-lysosomal glucosylceramidase. The value of these inhibitors as research tools in the elucidation of the physiological relevance of the non-lysosomal glucosylceramidase is discussed.

EXPERIMENTAL PROCEDURES

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Synthesis of Inhibitors

All reagents used for synthesis of the deoxynojirimycin derivatives were from Aldrich, except

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tetra-*O*-benzylglucopyranose, which was obtained from Sigma. Deoxynojirimycin (DNM)[2] was prepared from tetra-*O*-benzylglucopyranose according to the literature procedure (5). *N*-Propyl-, *N*-butyl-, *N*-pentyl-, and *N*-heptyl-DNM were prepared by literature procedure (6-8) by treatment of DNM HCl with the appropriate aldehyde under the agency of sodium cyanoborohydride and acetic acid. *N*-Pentanoyl-DNM was prepared by condensation of the known 2,3,4,6-tetra-*O*-benzyl-DNM (5) with valeryl chloride and subsequent hydrogenolysis of the benzyl ethers catalyzed by palladium on carbon. The *N*-acylated DNM derivatives containing adamantanemethyl, adamantanyl, phenantryl, cholesteryl, and β-cholestanyl substituents were prepared as follows. Glutaric anhydride was treated with 4-nitrophenol to afford pentanedioic acid mono-(4-nitrophenyl) ester. Treatment with oxalyl chloride under the agency of a catalytic amount of dimethylformamide afforded the corresponding 4-chlorocarbonylbutyric acid 4-nitrophenyl ester, which was subsequently condensed with adamantanemethanol, adamantanol, phenantrol, cholesterol, or β-cholestanol to afford 4-adamantanemethylcarbonylbutyric acid 4-nitrophenyl ester, 4-adamantanecarbonylbutyric acid 4-nitrophenyl ester, 4-phenantrylcarbonylbutyric acid 4-nitrophenyl ester, 4-cholesterylcarbonylbutyric acid 4-nitrophenyl ester, and 4β-cholestanylmethylcarbonylbutyric acid 4-nitrophenyl ester. Condensation of these esters with 2,3,4,6-tetra-*O*-benzyl-DNM and subsequent palladium/carbon-mediated hydrogenolysis afforded *N*-(4-adamantanemethanylcarboxy-1-oxo)-DNM, *N*-(4-adamantanylcarboxy-1-oxo)-DNM, *N*-(4-phenantrylcarboxy-1-oxo)-DNM, *N*-(4-cholesterylcarboxy-1-oxo)-DNM, and *N*-(4-β-cholestanylcarboxy-1-oxo)-DNM, respectively. Alternatively, the *N*-alkylated DNM derivatives containing adamantanemethyl and cholesteryl substituents were prepared by reduction of the known glutaric dialdehyde mono(diethyl)acetal to 5,5-diethoxypentan-1-ol (9), which was transformed to the corresponding methanesulfonic acid 5,5-diethoxypentyl ester by treatment with methanesulfonyl chloride and triethylamine. Condensation with adamantanemethanol and cholesterol, respectively, under the agency of sodium hydride and subsequent liberation of the aldehyde functionality afforded 5-(adamantan-1-yl-methoxy) pentanal and 5-(cholesteroloxy)pentanal, which were condensed with DNM under reductive amination conditions (sodium cyanoborohydride and acetic acid) to afford *N*-(5-adamantane-1-yl-methoxy)pentyl)-DNM (AMP-DNM) and *N*-(5-cholesteroxypentyl)-DNM (CP-DNM). The structures of these compounds are shown in Fig. 1.

Preparation of Spleen Extract and Membrane Suspension

Water extracts of Gaucher's disease and normal spleens were prepared by homogenization of 10 g of tissue in 30 ml of water (4 °C) using an Ultra-Turrax and centrifugation for 20 min at 15,000 × *g*. The membrane suspensions were prepared by resuspending the pellet in 30 ml of 50 mM potassium phosphate buffer (pH 5.8) and centrifugation (15 min, 15,000 × *g*). This procedure was repeated two times.

Enzyme Assays

All 4-methylumbelliferyl (4-MU) substrates used were obtained from Sigma. The activity of Ceredase® (Genzyme, Boston, MA) was determined with 3 mM 4-MU β-glucoside in the presence of 0.25% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in McIlvaine buffer (0.1 M citrate and 0.2 M phosphate buffer) (pH 5.2). The activity of the lysosomal glucocerebrosidase in splenic membrane suspensions was determined with 3 mM 4-MU β-glucoside in McIlvaine buffer (pH 5.2). The activity of the non-lysosomal glucosylceramidase in membrane suspensions was determined with 3 mM 4-MU β-glucoside in McIlvaine buffer (pH 5.8) upon preincubation for 30 min at room temperature with 2.5 mM conduritol B epoxide (CBE) (Sigma). The activity of the lysosomal α-glucosidase was measured with 0.3 mM 4-MU α-glucoside in 125 mM sodium acetate buffer

(pH 4.0). IC_{50} values were determined by variation of inhibitor concentrations. Assays were incubated at 37 °C and stopped by the addition of glycine/NaOH (pH 10.6). The amount of liberated 4-MU was determined with a Perkin-Elmer LS2 fluorometer.

In Vivo Inhibition Experiments

Melanoma Cells-- Human melanoma cells were cultured in RPMI 1640 medium (Flow Laboratories) supplemented with 5% fetal calf serum (Hyclone Laboratories). The enzyme activities were measured as described previously (4). In short, melanoma cells were incubated with 5 mM 4-MU β-glucoside in phosphate-buffered saline in the absence or presence of various DNM inhibitors. To distinguish between the contributions by both the lysosomal and non-lysosomal enzymes, the experiments were performed in parallel with melanoma cells that had been preincubated with and without CBE (2 h, 0.5 mM). The CBE-sensitive activity can be ascribed to the lysosomal glucocerebrosidase, and the CBE-insensitive activity to the non-lysosomal glucosylceramidase. After several time intervals, media samples were taken, and the fluorescence of the liberated 4-MU was measured.

Cultured Human Macrophages-- Human macrophages were obtained as described earlier (10). The deoxynojirimycin derivatives, dissolved in Me_2SO, were added to cultured macrophages at various concentrations by dilution in culture medium. It was checked that the minor amounts of Me_2SO introduced in this manner were without effect. After 4 days of preincubation with the inhibitors, *in situ* enzyme activities were measured using fluorescent lipid substrates. For glucosylceramidase and glucocerebrosidase activity measurement, C_6-NBD-glucosylceramide was used as substrate, and glucosylceramide synthase activity was determined with C_6-NBD-ceramide as substrate. The lipid substrates were complexed to fatty acid-free bovine serum albumin at a 1:1 molar ratio (11). The cells were preincubated for 2 h with or without 300 μM CBE, washed, and incubated for 1 h with 3 ml of medium with or without 300 μM CBE and 5 nmol of the substrate-bovine serum albumin complex. The cells were harvested, lipids were extracted, and the C_6-NBD lipids were separated by thin-layer chromatography (11). The lipids were quantified with a luminescence spectrometer (Perkin-Elmer LS50). Enzyme activities were related to those in the absence of the inhibitor.

Synthesis of C_6-NBD-glucosylceramide

C_6-NBD-glucosylceramide was synthesized as described (12). Briefly, glucosylsphingosine (2.17 μmol) and C_6-NBD-hexanoic acid succinimidylic ester (4.33 μmol) (both from Sigma) were dissolved in 530 μl of dimethylformamide. Upon addition of 20 μl of diisopropylethylamine, the mixture was stirred at 30 °C for several hours. Synthesis was checked by analysis on a thin-layer plate (developing system of chloroform/methanol/water (65:25:4 by volume)) using ultraviolet illumination and iodine. The reaction mixture was diluted with methanol, evaporated under nitrogen, and analyzed on several thin-layer plates. The separated C_6-NBD-glucosylceramide was scraped off and extracted with chloroform/methanol (1:1 by volume), chloroform/methanol (2:1 by volume), and methanol. The supernatants were collected, evaporated, and applied to Lichroprep RP-18 columns as described (11). C_6-NBD-glucosylceramide was eluted with methanol and chloroform/methanol (1:1 by volume), evaporated to dryness, and dissolved in ethanol. The concentration was determined spectrophotometrically (485 nm, ε = 20,000 units/mol/liter) and fluorometrically (excitation at 480 nm and emission at 530 nm).

Density Gradient Electrophoresis

Melanoma cells were cultured as described above, and a crude microsome fraction was prepared from a post-nuclear supernatant exactly as described earlier (13).

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Design of a Specific Inhibitor for the Non-lysosomal Glucosylceramidase Activity-- In a previous study (4), a number of known glucosidase inhibitors (D-gluconolactone, castanospermine, deoxynojirimycin, and *N*-butyldeoxynojirimycin) were tested with respect to their capacity to inhibit the non-lysosomal glucosylceramidase. Although deoxynojirimycin was found to be a potent inhibitor, its value was limited because, even at low concentrations, it inhibited not only the non-lysosomal glucosylceramidase, but also the lysosomal glucocerebrosidase (4). Previous research has also revealed that the non-lysosomal glucosylceramidase is tightly integrated in the membrane and hydrolyzes its substrate glucosylceramide while it is also inserted in the membrane (4). These findings prompted us to develop novel, more specific inhibitors for the non-lysosomal glucosylceramidase, assuming that the desired inhibitor should contain a deoxynojirimycin moiety, an *N*-alkyl spacer, and a large hydrophobic group, promoting correct insertion in the membrane. To test this concept, a series of deoxynojirimycin derivatives was synthesized as described under "Experimental Procedures" (Fig. 1).

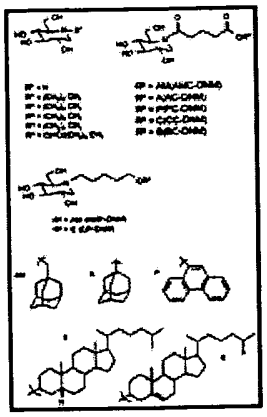

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Fig. 1. Nomenclature of the developed inhibitors. The structure formulas of the deoxynojirimycin-type inhibitors are depicted. *AM*, adamantanemethyl; *A*, adamantanyl; *P*, phenantryl; *C*, cholesteryl; *B*, β-cholestanyl.

In Vitro Inhibition-- The inhibitory capacity and specificity of the deoxynojirimycin-based compounds were examined by analysis of their effects on the activity of purified human lysosomal glucocerebrosidase (Ceredase) and α-glucosidase and on the activity of the lysosomal glucocerebrosidase and the non-lysosomal glucosylceramidase as present in a membrane suspension prepared from human spleen. Under "Experimental Procedures," the sources of the enzyme preparations and the activity measurements are described.

Table I gives an overview of the apparent IC_{50} values of the various enzymes for the deoxynojirimycin derivatives. It can be seen that inhibition of the non-lysosomal glucosylceramidase by the *N*-alkyl derivatives of deoxynojirimycin increased with increasing chain length. Furthermore, it was found that the presence of a carbonyl moiety (*i.e.* an *N*-acyl spacer) in the spacer negatively influenced the inhibitory capacity.

Table I
Apparent IC_{50} values of various glycosidases

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IC_{50} values (*i.e.* inhibitor concentration resulting in 50% inhibition) were determined by variation of inhibitor concentrations. Assays were performed as described under "Experimental Procedures." All constants are expressed in micromolar. The K_m for 4-MU β-glucoside is expressed in millimolar.

Addition of a large hydrophobic group such as adamantane (AMP-DNM) or cholesterol (CP-DNM) to an *N*-pentyl spacer dramatically increased the capacity to inhibit the glucosylceramidase activity. The apparent IC_{50} values for AMP-DNM and CP-DNM are extremely low: 2 nM and 0.1 μM, respectively. For a comparison, the IC_{50} values for DNM and butyl-DNM are 30 and 0.3 μM, respectively.

Table I shows that the lysosomal glucocerebrosidase is, in general, less sensitive to inhibition by deoxynojirimycin derivatives than the non-lysosomal glucosylceramidase. Pure glucocerebrosidase in solution and the same enzyme associated with splenic membranes show a different sensitivity to the inhibitors. Apparently, the kinetic properties of glucocerebrosidase in these two different states differ, as is also suggested by the clear difference in apparent K_m for 4-MU β-glucoside. Both the purified soluble and the membrane-associated lysosomal glucocerebrosidases are most potently inhibited by deoxynojirimycin analogues with an *N*-pentyl spacer with a coupled large hydrophobic group (Table I). With respect to the lysosomal α-glucosidase, it was found that variation of the bulky substituent in the *N*-alkyl series, in general, exerted relatively little effect. However, the compounds *N*-(4-adamantanemethanylcarboxy-1-oxo)-DNM, *N*-(4-adamantanylcarboxy-1-oxo)-DNM, *N*-(4-phenantrylcarboxy-1-oxo)-DNM, *N*-(4-cholesterylcarboxy-1-oxo)-DNM, and *N*-(4β-cholestanylcarboxy-1-oxo)-DNM were very poor inhibitors (Table I).

In Vivo Inhibition-- The capacity of deoxynojirimycin analogues to inhibit the non-lysosomal glucosylceramidase and glucocerebrosidase activities in cultured melanoma cells was investigated. For this purpose, cells were incubated with 4-MU β-glucoside, and its hydrolysis by the two enzymes was determined. To distinguish between the contributions by both enzymes, the experiments were performed in parallel with melanoma cells that had been preincubated either with or without CBE. The CBE-sensitive activity can be ascribed to the lysosomal glucocerebrosidase, and the insensitive activity to the non-lysosomal glucosylceramidase. Table II shows that again the most potent inhibitors were found to be AMP-DNM and CP-DNM. The non-lysosomal glucosylceramidase was very sensitive to inhibition, even more pronounced than in cell homogenates. For intact cells, the apparent IC_{50} values of AMP-DNM and CP-DNM were ~0.3 and 50 nM, respectively. At these concentrations, no significant inhibition of the lysosomal glucocerebrosidase activity was detectable (Table II).

Table II
In vivo inhibition by deoxynojirimycin analogues

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Melanoma cells were incubated with various concentrations of inhibitors to determine their IC_{50} values. Activities of glucosylceramidase and glucocerebrosidase were determined as described (4).

Next, the effects of AMP-DNM and butyl-DNM were also examined with a more physiological lipid substrate in macrophages, the cells involved in glucosylceramide storage in Gaucher's disease. The activity of the

lysosomal glucocerebrosidase and the non-lysosomal glucosylceramidase were measured using C_6-NBD-glucosylceramide as substrate. After incubation of the cells with the substrate, lipids were extracted and separated by thin-layer chromatography, and the various fluorescently labeled metabolites were fluorometrically quantified. Again, CBE was employed in these experiments to discriminate between the metabolism due to the action of the non-lysosomal glucosylceramidase and glucocerebrosidase. The result of one of these experiments is depicted as an example in Fig. 2. It has to be mentioned that no degradation of C_6-NBD-ceramide was detected, as observed in earlier studies (4). Apparently, C_6-NBD-ceramide is a poor substrate for the lysosomal ceramidase, or it leaves the lysosomes prior to degradation.

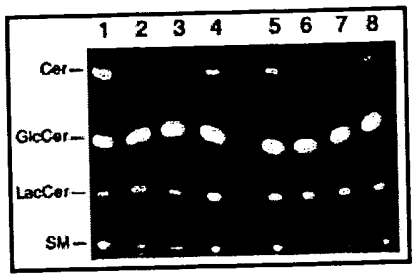

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Fig. 2. Inhibition of the non-lysosomal glucosylceramidase by AMP-DNM. Cultured human macrophages were treated with AMP-DNM, and the enzyme activities of glucocerebrosidase and the non-lysosomal glucosylceramidase were measured using the C_6-NBD-glucosylceramide substrate. Lipids were extracted from the cells and analyzed by TLC as described under "Experimental Procedures" and in Ref. 4. A control incubation without CBE is shown in *lane 1*; in all other lanes, the cells were preincubated with CBE. The following concentrations of AMP-DNM were added to the cells: 1 nM (*lanes 2* and *6*), 0.05 nM (*lanes 3* and *7*), 0.0025 nM (*lanes 4* and *8*). No inhibitor was added to the cells in *lane 5*. The cells in *lanes 2-4* were treated with AMP-DNM for 1 h after 5 days in culture and were subsequently cultured for 9 days in the absence of AMP-DNM; the cells in *lanes 6* and *7* were treated with AMP-DNM continuously starting after 5 days in culture, and new inhibitor was added after media changes. Markers are indicated: *Cer*, ceramide; *GlcCer*, glucosylceramide; *LacCer*, lactosylceramide; *SM*, sphingomyelin.

The inhibition of the activities of the lysosomal glucocerebrosidase and the non-lysosomal glucosylceramidase by the tested deoxynojirimycin analogues in macrophages was comparable to that in melanoma cells (Table III). Incubation of macrophages with 0.05-1 nM AMP-DNM led to marked inhibition of the non-lysosomal glucosylceramidase activity, whereas the lysosomal glucocerebrosidase activity was not decreased under these conditions. In fact, we repeatedly noted a slight increase in the activity of the lysosomal glucocerebrosidase in cells treated with the inhibitor. Most likely, this is due to the fact that, upon inhibition of the non-lysosomal enzyme, more substrate reached the enzyme in the lysosomal compartment. Up-regulation of the lysosomal glucocerebrosidase in cells pretreated with inhibitor seems unlikely since enzyme activity was found to be increased only in intact macrophages and not in homogenates of the same cells.

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Table III
Effect of DNM analogues on cultured macrophages

Human macrophages, obtained and cultured as described (10), were incubated with different concentrations of butyl-DNM or AMP-DNM. After 4 days of preincubation with inhibitor, glucosylceramidase and glucocerebrosidase activities were determined with C_6-NBD-glucosylceramide as substrate (4). Enzyme activities are related to those in the absence of inhibitor (100%).

Because of the extreme sensitivity of the non-lysosomal glucosylceramidase to AMP-DNM, we investigated whether the inhibition by this compound is fully reversible. To test this, macrophages were either treated with

AMP-DNM for 1 h and subsequently washed extensively and cultured for 9 days in the absence of the inhibitor or long-term treated with the inhibitor. Next, the activity of the non-lysosomal glucosylceramidase was determined with C_6-NBD-glucosylceramide as substrate. It was observed that the 1-h treatment with inhibitor led to a complete inhibition of the glucosylceramidase activity for at least 9 days, suggesting that the inhibitor is not easily removed from the enzyme or its surrounding membrane (Fig. 2).

Subcellular Localization of the Non-lysosomal Glucosylceramidase-- The easy access of hydrophobic AMP-DNM to the non-lysosomal glucosylceramidase in intact cells made us to look more closely into the localization of the enzyme using a recently developed subcellular fractionation technique that is a combination of density gradient centrifugation and free-flow electrophoresis (13, 14). As shown in Fig. 3, the non-lysosomal glucosylceramidase was recovered in fractions 30-42 of the gradient, which are known to contain light endosomal structures. The apparent localization of the enzyme in compartments close to the cell surface might explain its relatively high sensitivity to inhibition by AMP-DNM in intact cells as compared with cell homogenates. Presumably, in homogenates, a much larger proportion of the lipophilic inhibitors is scavenged by membrane fragments that do not contain the enzyme compared with incubation of intact cells with the compounds.

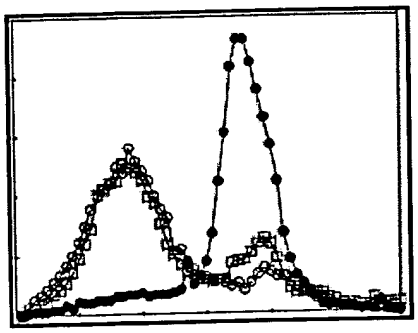

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Fig. 3. Density gradient electrophoresis profile of the different glucosylceramide-hydrolyzing enzymes. Density gradient electrophoresis was performed as described under "Experimental Procedures." The enzyme activities of β-hexosaminidase (lysosomal marker) (□), glucocerebrosidase (○), and the non-lysosomal glucosylceramidase (•) were measured in the fractions.

Specificity of AMP-DNM as an Inhibitor of the Non-lysosomal Glucosylceramidase-- We studied to what extent other reactions were also inhibited upon incubation of intact cells with AMP-DNM. Glucosylceramide synthase is a glucosyltransferase that catalyzes the synthesis of glucosylceramide from ceramide and UDP-glucose. The enzyme has been reported to be particularly sensitive to inhibition by butyl-DNM (6). Glucosylceramide synthase activity was measured by incubation of intact cells with C_6-NBD-ceramide and analysis of C_6-NBD-glucosylceramide formation. In this manner, it was found that, in agreement with the previous report (6), the IC_{50} value of butyl-DNM is ~25 μM and, furthermore, that the IC_{50} of AMP-DNM is 25 nM. In addition, it was observed that incubation of melanoma cells and macrophages with 1 nM AMP-DNM did not result in a significant reduction of glucosylceramide synthase activity, whereas concomitantly, the non-lysosomal glucosylceramidase was almost completely inhibited.

The trimming α-glucosidases in the endoplasmic reticulum are also known to be sensitive to hydrophobic deoxynojirimycin analogues. The effect of AMP-DNM in this respect was examined by studying the folding of influenza hemagglutinin in the endoplasmic reticulum, as described (15). No inhibitory effect of AMP-DNM at 1 nM on oligosaccharide chain modification that resulted in delayed folding of influenza hemagglutinin (16) was

detectable (data not shown). Only at concentrations above 0.2 mM AMP-DNM was clear inhibition of this process detected.

DISCUSSION

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Our investigation has led to the generation of potent inhibitors of the non-lysosomal glucosylceramidase. In particular, AMP-DNM is an attractive compound in this respect. A complete inhibition of non-lysosomal glucosylceramidase activity occurs upon incubation of intact cells with extremely low concentrations of the DNM derivative. The localization of the enzyme close to the cell surface, the design of the compound, and its tendency to associate with membranes probably all contribute to this. At low nanomolar concentrations, AMP-DNM seems not to significantly affect other enzyme systems that are sensitive to hydrophobic deoxynojirimycin analogues, such as the glucosylceramide synthase and oligosaccharide chain-trimming glucosidases. The compound should therefore be useful for investigations on the non-lysosomal glucosylceramidase. It will be of particular interest to study the extent to which the enzyme activity is relevant for the lipid metabolism coupled to signal transduction processes. Hypothetically, the enzyme could indirectly affect the activity of neutral sphingomyelinase by changing the concentration of ceramide. Alternatively, the enzyme itself might be involved in the conversion of some extracellular signal into increased ceramide concentrations and corresponding signaling.

Our attempts to purify the non-lysosomal glucosylceramidase by conventional purification procedures have been unsuccessful so far. A major complication is caused by the instability of the enzyme upon solubilization with detergents. On the basis of the findings made in this study, it seems attractive to exploit the interaction of hydrophobic deoxynojirimycin analogues with the non-lysosomal glucosylceramidase for affinity purification of the enzyme. Previously, the lysosomal glucocerebrosidase and the endoplasmic reticulum α-glucosidase have been purified on *N*-alkyldeoxynojirimycin derivatives immobilized on a column matrix (17, 18). The feasibility of a comparable approach for the non-lysosomal glucosylceramidase is currently being studied.

Another important application for the inhibitors may be found in the field of Gaucher's disease. In this disorder, tissue macrophages store glucosylceramide due to the inherited deficiency in lysosomal glucocerebrosidase activity (3). The abnormal lipid-laden macrophages, called Gaucher's cells, are thought to be an essential factor in the pathophysiology of the disease.[3] These cells most likely secrete cytokines and hydrolases that promote tissue turnover and propagate the formation of novel storage macrophages. The mechanism by which impaired lysosomal glucosylceramide degradation leads to activation of the storage cells is unknown. It is conceivable that the non-lysosomal glucosylceramidase plays an important role in the process. Elevated concentrations of glucosylceramide in macrophages of glucocerebrosidase-deficient individuals might lead to increased activity of the non-lysosomal glucosylceramidase. Thus, ceramide formation could be constitutively increased in membranes close to the cell surface, affecting signal transduction pathways and promoting the characteristic activation state of Gaucher's cells. The newly developed inhibitors should allow studies to be performed on the importance of the non-lysosomal glucosylceramidase in this respect. If this enzyme activity indeed proves to be an essential factor in the pathogenesis of Gaucher's disease, one might consider the use of the inhibitors in therapeutic intervention of the disorder. Presently, Gaucher's disease is treated by regular intravenous infusions with large amounts of a modified human glucocerebrosidase (Ceredase) (19, 20). This enzyme supplementation therapy is very successful; however, the application is restricted due to the high costs. Interestingly, the use of butyl-DNM as a therapeutic agent for Gaucher's disease has already been considered for a completely different

reason. It has been argued that a marked inhibition of the synthesis of glucosylceramide may be beneficial for Gaucher's disease patients since this would result in a reduction in the amount of glucosylceramide that has to be degraded by macrophages. A number of inhibitors of glucosylceramide synthase have been proposed in connection with this so-called substrate deprivation approach, including 1-phenyldecanoylamino-3-morpholino-1-propanol and its analogue 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (21) and, more recently, butyl-DNM (6, 22) and the galactose analogue, butyldeoxygalactonojirimycin (23). The results of our investigation indicate that administration of butyl-DNM will inhibit not only glucosylceramide synthase, but also the non-lysosomal glucosylceramidase, which is, in fact, much more sensitive to this inhibitor. For deoxygalactonojirimycin and *N*-butylgalactonojirimycin, for which inhibition of the glucosylceramide synthase was also recently demonstrated, we obtained similar results as for DNM and butyl-DNM in the same concentration range (data not shown), indicating that the non-lysosomal glucosylceramidase can also be inhibited by these compounds. It can be envisioned that the combined inhibition by hydrophobic deoxynojirimycin analogues of glucosylceramide synthase and the non-lysosomal glucosylceramidase activities in Gaucher's patients might act as a double-edged sword since this could reduce the formation of storage cells and inhibit the deleterious activation of these cells. In conclusion, the newly developed hydrophobic deoxynojirimycin derivatives, in particular AMP-DNM, have proven to be extremely potent inhibitors of the non-lysosomal glucosylceramidase and should be valuable research tools in the elucidation of the physiological role of this enzyme.

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FOOTNOTES

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The abbreviations used are: DNM, deoxynojirimycin; AMP-DNM, *N*-(5-adamantane-1-yl-methoxy)pentyl)-DNMCP-DNM, *N*-(5-cholesteroxypentyl)-DNM4-MU, 4-methylumbelliferylCBE, conduritol B epoxideC_6-NBD, 6-(*N*-7-nitrobenz-2-oxa-1,3-diazol-4-ylaminocaproyl).

[1] Although the terms glucosylceramidase and glucocerebrosidase can, in principle, both be used for an enzyme that hydrolyzes glucosylceramide (=glucocerebroside), we use the common term glucocerebrosidase to indicate

the CBE-inhibitable lysosomal enzyme that is deficient in Gaucher's patients, and glucosylceramidase for the CBE-insensitive non-lysosomal enzyme that is not deficient in Gaucher's patients.

[3] J. M. F. G. Aerts, R. G. Boot, G. H. Renkema, M. Verhoek, S. van Weely, C. E. M. Hollak, M. H. J. van Oers, A. Erikson, and H. Michelakakis, submitted for publication.

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